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13. SUPPLEMENTARY NOTES

14. ABSTRACT The purpose of this research is to identify the optimum combination of growth factors that stimulate cartilage regeneration across a critical size defect (CSD) in the fibula, using the axolotl, Abystoma mexicanum as a model system. The scope of the research is to characterize combinations of soluble factors (growth factors and tissue extracts) absorbed into a standard scaffold of pig small intestine submucosa (SIS). From October 2011 to April 2012, we determined the CSD as slightly less than 40% the length of the fibula.fracture, characterized the response to fracture and defects of various sizes, and identified 7 growth factors involved in fracture repair by bioinformatic analysis. We determined that regeneration takes place over gaps of up to 20%, but not at 40 and 50% and that SIS scaffold alone does not promote regeneration. In April 2013 we presented a poster summarizing this data at the Experimental Biology meeting in Boston. Since then, we have been testing the ability of a number of growth factor combinations, blastema extract, and limb tissue extract to promote cartilage regeneration across 50% gaps (20% greater than the actual CSD). Three sets of factors promoted such regeneration by 3 months post-implant, the growth factor combinations BMP4/VEGF and BMP4/HGF, and protein extract of intact limb tissue, of which the latter two gave the best results. We determined that the best way to image the regenerating tissue was by whole mount methylene blue staining and routine H&E histology, reserving micro-CT imaging for bone only. We think BMP4/HGF and tissue extract were successful because they initiated the whole cascade of events required for cartilage development. These results indicate that the axolotl fibula can be used as an in vivo screening system for soluble factors (as well as scaffolds) that promote cartilage regeneration across a CSD.

15. SUBJECT TERMS
Regeneration across a critical size defect in axolotl fibula, efficacy of growth factor combinations

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Introduction

The subject of our research is development of the urodele salamander Ambystoma mexicanum as a model to find the optimum set of growth factors to enable regeneration of a cartilage template across a critical size defect (CSD) in the long bones of the extremities. Unlike most models that attempt to regenerate bone directly, with less than optimum filling, regeneration of a cartilage template mimics the initial step of both the normal development of a long bone and fracture repair. The axolotl can be used to screen single molecules or combinations of molecules for their ability to stimulate regeneration across a CSD. This model has advantages over mammalian CSD models in that there is greater ease of surgical operation and tissue processing, no requirement for bone fixation, and is less expensive to maintain[1]. Our purpose is to identify the optimum combination of growth factors that simulate cartilage regeneration across a CSD (and ultimately, the optimum scaffold to deliver these factors). The scope of our research includes defining the CSD for a long bone of the lower leg, the anatomical and histological characterization of cartilage and bone regeneration over defects less than, and equal to or greater than, the CSD, defining protein release characteristics from a standard SIS delivery scaffold, identifying the optimum growth factor combination to be delivered through biochemical analysis of axolotl limb and regeneration blastema protein extracts and by bioinformatic techniques, and testing/verifying the effectiveness of this combination by delivery in the appropriate scaffold.

Body

1. Results of Implanting SIS scaffold Alone

We confirmed that no intrinsic regeneration takes place in a 50% CSD of the fibula, and that the SIS scaffold used as a growth factor delivery vehicle did not promote regeneration across a 50% CSD.

2. Selection of Growth Factor Combinations

During the first quarter, we identified, by bioinformatics mining[2] of the literature on fracture repair, eight growth factors to test in four combinations for their ability to promote regeneration across a 50% CSD. The growth factors were: VEGF-A, HGF, FGF-2, TGFβ-3, BMP4, PDGF-A, EGF, and PDGF-B. All of the commercial growth factors available, except EGF, had an amino acid sequence homology to the corresponding Xenopus growth factors (the closest amphibian to the axolotl for which such data were available) of 64% or greater. EGF was eliminated from the experiments because of its low homology (41%), as was PDGF-B (homology = 39%). Six combinations of the remaining six growth factors, along with limb blastema protein extract and intact limb tissue extract, were tested for their ability to promote regeneration across a 50% CSD. We used a physical maceration and cell lysis procedure to extract a high concentration of protein (5.76 mg/ml) from blastema and tissue cells. These extracts induced no inflammatory reaction when injected into the muscle of the tibia-fibula.

3. Testing Growth Factor Combinations

During the second quarter, we focused on setting up the experiments to test growth factor combinations and protein extracts on CS defects of 50%. Lengths of scaffold equaling the size of the defect were soaked in 50 ul solutions of the growth factor combinations overnight at 4^{o}C. Stock solutions of the growth factor combinations were made by centrifuging the growth factors at 13,000 rpm and re-suspending the pellet in amphibian PBS to obtain concentrated solutions. Then aliquots of individual growth factors were combined to obtain 200 μl of each combination at the concentrations shown in Table 1. Growth factor concentrations were decided by reference to the published literature on the use of each factor for in various biological systems, including wound repair and bone regeneration. To absorb the growth factor combinations into the scaffolds, lengths of scaffold equivalent to half the length of the fibula were cut, and five scaffolds at a time were immersed in 50μl of growth factor solution contained in a small plastic well made from the inverted cap of an Eppendorf tube. The scaffolds were allowed to absorb the growth factor soultion overnight at 4^{o}C and then

were implanted into 50% segment defects made in the fibula, under 0.08% MS-222 anesthesia. Incisions were closed with silk suture and the animals kept in analgesic-strength (0.02%) MS-222 overnight before returning to maintenance water the next day. In addition, we injected (under anesthesia) the polychrome dyes calcein (15μg/gm body wt), alizarin complexone (30μg/gm body wt), and calcein blue (30 μg/gm body wt) into the operated limbs to enable visualization of any osteogenesis.

Eight groups of implants were made to 50% segment defects in the fibulas of axolotls that averaged 14 cm in snout-tail tip length (**Table 1**). Six of the groups were different growth factor combinations, and two were protein extracts of mid-late bud regeneration blastemas, and intact limb tissues, respectively.

Table 1

Treatment Group	GF Concentration	# of animals
1[a]. Amphibian PBS	---	10
2[b]. BMP-4	10 ng/μl	10
VEGF	25 ng/μl	
3. BMP-4	10 ng/μl	10
HGF	10 ng/μl	
4. BMP-4	10 ng/μl	10
VEGF	25 ng/μl	
HGF	10 ng/μl	
5. BMP-4	10 ng/μl	10
VEGF	25 ng/μl	
HGF	10 ng/μl	
FGF-2	8 ng/μl	
6. BMP-4	10 ng/μl	10
VEGF	25 ng/μl	
HGF	25 ng/μl	
FGF-2	8 ng/μl	
EGF	10 ng/μl	
TGFβ-3	2ng/μl	
PDGF-AA	10 ng/μl	
7. Blastema protein extract	7μg/μl	10
8. Limb protein extract	6μg/μl	10

[a]Negative control
[b]Positive control from Reference 15.

4. Results of Growth Factor Treatments

During Quarter 3, we harvested implanted limbs that had been regenerating for two months with 10% and 20% gaps. These limbs had been injected with three fluorochrome dyes for live imaging to detect bone regeneration. Small amounts of bone regeneration were detected at the proximal and distal ends of 50% gaps treated by scaffold implant with VEGF/BMP4 (Figs 1, 2).

During Quarter 4, we have sectioned three-month samples of 50% defects and stained the sections with H&E to assess regeneration. We detected regeneration in three sets of samples: BMP4/VEGF-treated, BMP4/HGF-treated, and the limb tissue extract-treated samples (Figs 3-8). Of the three groups, the combination BMP4/HGF resulted in the most substantive regeneration, nearly the whole length of the gap. Interestingly, regeneration appears to be polarized, extending from the proximal stump of the fibula. These findings allow us to now focus on these three treatment groups. Going forward, we will examine examine 4 and 5-month samples by methylene blue stain for whole mounts and trichrome staining of sections. We will also set up a new group of implants treated with the successful factor combinations factors.

We will also be changing our scaffold strategy, and reducing the size of the gap to approach the actual CSD size. Our defect size of 50% is ~20% greater than the actual critical size defect of slightly less than 40% that we established earlier. We feel that the 40% gap is a more realistic challenge for the system, since we have found that in some cases, the bone stumps dissolve on one or both ends. Because the regeneration is polarized, it may be that an intact bone stump is necessary for the initiation of regeneration. Another problem is that in either a 40% or 50% size gap, the distal end of the fibula often becomes deflected at an angle to the proximal end. The braided SIS scaffold we have been using is not stiff enough to keep the ends of the fibula aligned; it unravels when hydrated by body fluids. We have obtained a rod form of SIS that we think will maintain better alignment and will re-test a polycaprolactone scaffold synthesized by Dr. Bottino. In previous tests of this scaffold, freshly synthesized scaffold was rejected from the limb, probably because of an inflammatory reaction to some of the solvents used in the synthetic process. This scaffold will be re-tested after washing with several changes of PBS over two days.

Key Research Accomplishments

- Determined that long-term implants of SIS scaffold alone do not promote regeneration across a 50% gap in the axolotl fibula
- Determined that the growth factor combination of bone morphogenetic protein 4 and hepatocyte growth factor, and protein extract from intact limb tissue both promote regeneration within a 50% defect.
- Revealed shortcomings of braid SIS, leading to better delivery system of rod SIS scaffold and tubular polycaprolactone scaffold.

Reportable Outcomes

Poster and Abstract: Experimental Biology, April 22, 2013, Boston MA

Characterization of Segment Defect Regeneration in the Axolotl Fibula

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We determined the critical size defect (CSD) for regeneration of the axolotl fibula by surgically removing bone segments comprising 10, 20, 40, and 50% of the length of the fibula in 15-20 cm axolotls. The limbs were first subjected to X-ray and micro-CT imaging. Half the specimens were stained with methylene blue/alizarin red for cartilage/bone and the other half sectioned and stained with H&E. H&E staining revealed initiation of cartilage formation in 10% and 20% defects by one month; cartilage and bone were regenerated by three months. None of the 40% defects regenerated cartilage by two months post-operation, and 7/8 (87.5%) failed to regenerate cartilage at three months. None of the 50% defects regenerated cartilage after three months. H&E staining showed that the 40% and 50% defects filled in with fibrous soft tissue. We concluded that the CSD is slightly less than 40% of the length of the fibula. A pig small intestine submucosa (SIS) scaffold did not promote regeneration across a 50% defect. The scaffold degraded by two months after implantation while the defect was filled by connective tissue and disorganized muscle fibers. Our aim is to use the axolotl fibula as a new, convenient model to screen for the optimum combination of growth factors and scaffolds that promote cartilage formation in a CSD, thus initiating the cascade of events in normal endochondral bone development.

Research supported by the Telemedicine & Advanced Technology Research Center, US Army Medical Research and Materiel Command, Award # W81XWH-11-1-0743

Conclusion

Both BMP-4/HGF and intact limb tissue protein extract promote cartilage regeneration across a greater than CSD in the axolotl fibula, whereas other more complex combinations of growth factors, as well as regeneration blastema extract fail to do so. BMP-4/HGF trigger the initial events of a cascade that leads to cartilage formation, most likely by recruitment of MSCs cells from the periosteum of the adjacent tibia or fibroblasts from muscle and dermis.

References

1. Song F., et al., *Organogenesis* ***6***, 141 (2010).

2. Palakal M., et al., *Proc IEEE Comput Soc Bioinform Conf* **1**, 97 (2002).

Appendix

None

Supporting Data

Powerpoint Figures 1-8. Figs. 3-8 are longitudinal sections stained with H&E and photographed at 2.5X.

Legends for Figures

Fig. 1: PBS-treated (A) and 50% CSD sample (B) treated with VEGF/BMP4 and injected successively with calcein (green), alizarine (red) and calcein blue. Harvested at 2 months post-surgery and treatment. There was very little or no bone regeneration during the first two weeks in the PBS-treated animal (green, red and blue overlap). Bone regeneration occurred in the VEGF/BMP4 sample (separation of green, red and blue).

Fig. 2: Micro-CT image of 50% CSD treated with VEGF/BMP4 and harvested at three months post-surgery. Bone regeneration had taken place from the distal cut end of the fibula (to the left).

Fig. 3. 50% fibular defect treated with BMP4/VEGF/HGF/FGF2/PDGFAA/TGF beta-3, three months post-implant. A small amount of regenerated cartilage (RC) was present at the proximal end of the fibula (PF). The distal end of the fibula (PF) was severely angled with respect to the proximal end. A small amount of cartilage appears to have regenerated transversely on the distal fibula stump (asterisk). Lines indicate the boundaries of the gap, within which appear to be remnants of the SIS scaffold. The adjacent tibia is indicated by the long arrow.

Fig. 4. Regeneration of cartilage across a 50% defect (areas enclosed within the rectangles) three months after treatment with BMP4/HGF. The cartilage is irregular in shape and is surrounded by a thin periosteal bone shell (blue arrow). Black arrow runs the length of the adjacent tibia. Proximal end of the tibia to the left. Note the muscle mass (black star) attached to the distal end of the regenerating cartilage.

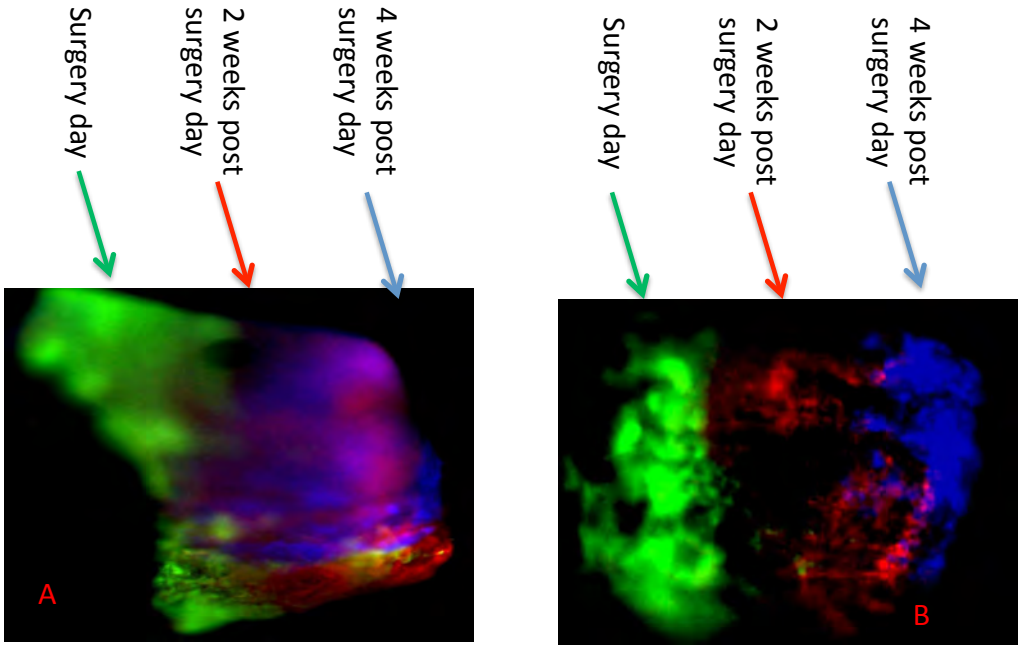
Fig. 5. 50% defect three months after treatment with BMP4/HGF. In this case, an irregular secondary length of cartilage (orange arrow) was induced along the axis of the tibia (Black arrow). Vertical lines indicate the boundaries of the gap in the fibula. The distal tibia has been resorbed except for a nodule of cartilage. PT = proximal tibia; PF = proximal fibula stump.

Fig. 6. Another example of a 50% defect three months after treatment with BMP4/HGF. Cartilage is regenerating (asterisk) from the proximal stump of the tibia (PF).

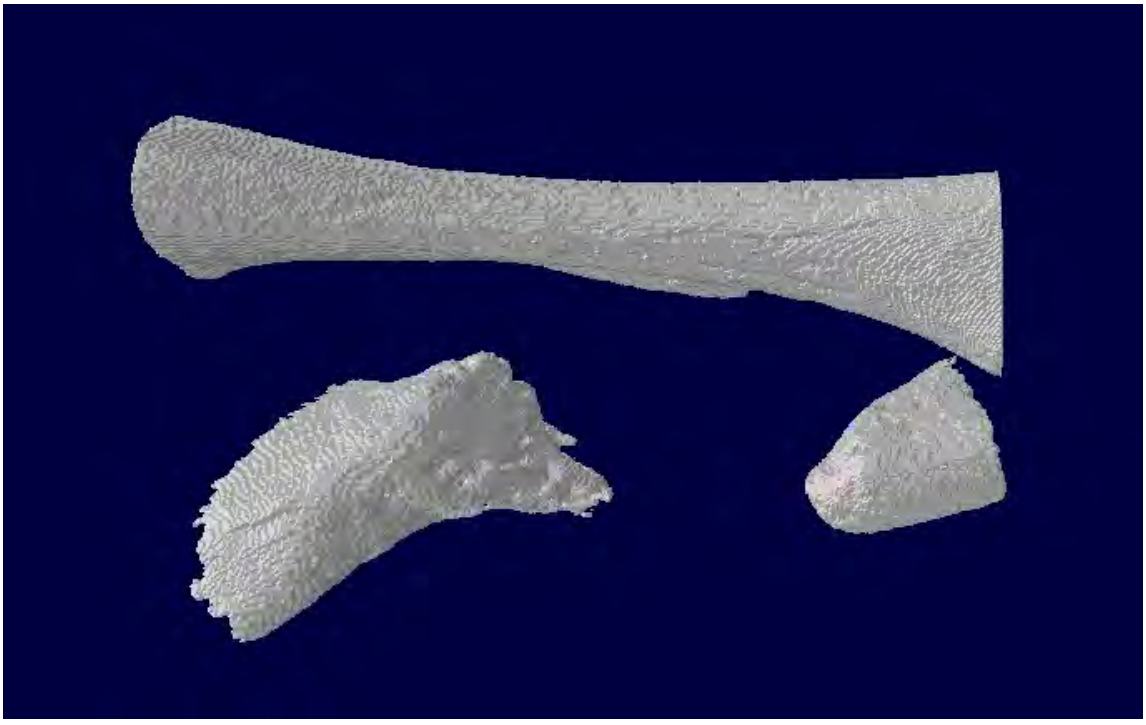
Fig. 7: 50% defect three months after treatment with intact limb tissue extract. Vertical lines indicate the boundaries of the defect. Regenerated cartilage (green star) formed under the skin off to the side of the gap. There appears to be ossification in the center and development of a marrow cavity.

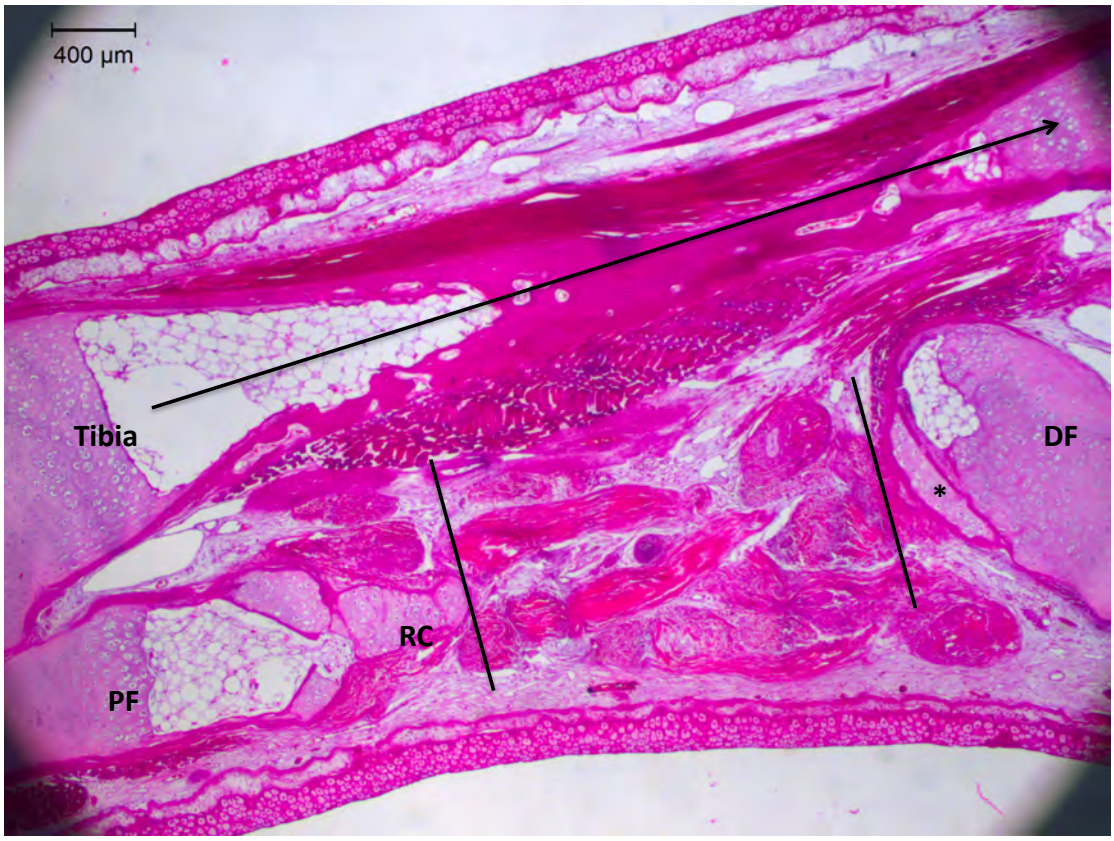
Fig. 8: 50% defect three months after treatment with BMP4/VEGFB. An irregular tongue of cartilage (asterisk(is regenerating from the distal end of the fibula (DF). The gap (green star) is filled with remnants of the SIS scaffold.

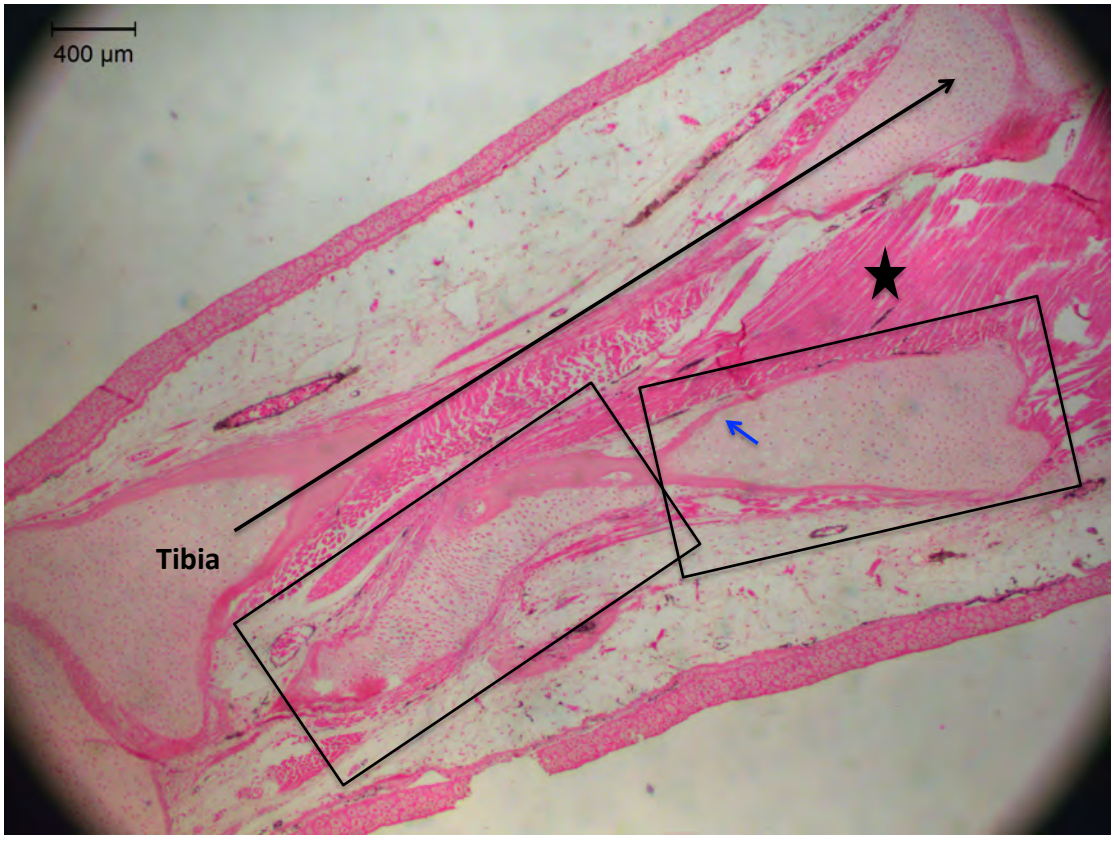
Negative control and VEGF+BMP4 treatment of 50% CSD at 2 months post-surgery



50% CSD treated with VEGF/BMP4




400 µm
Tibia
DF
*
RC
PF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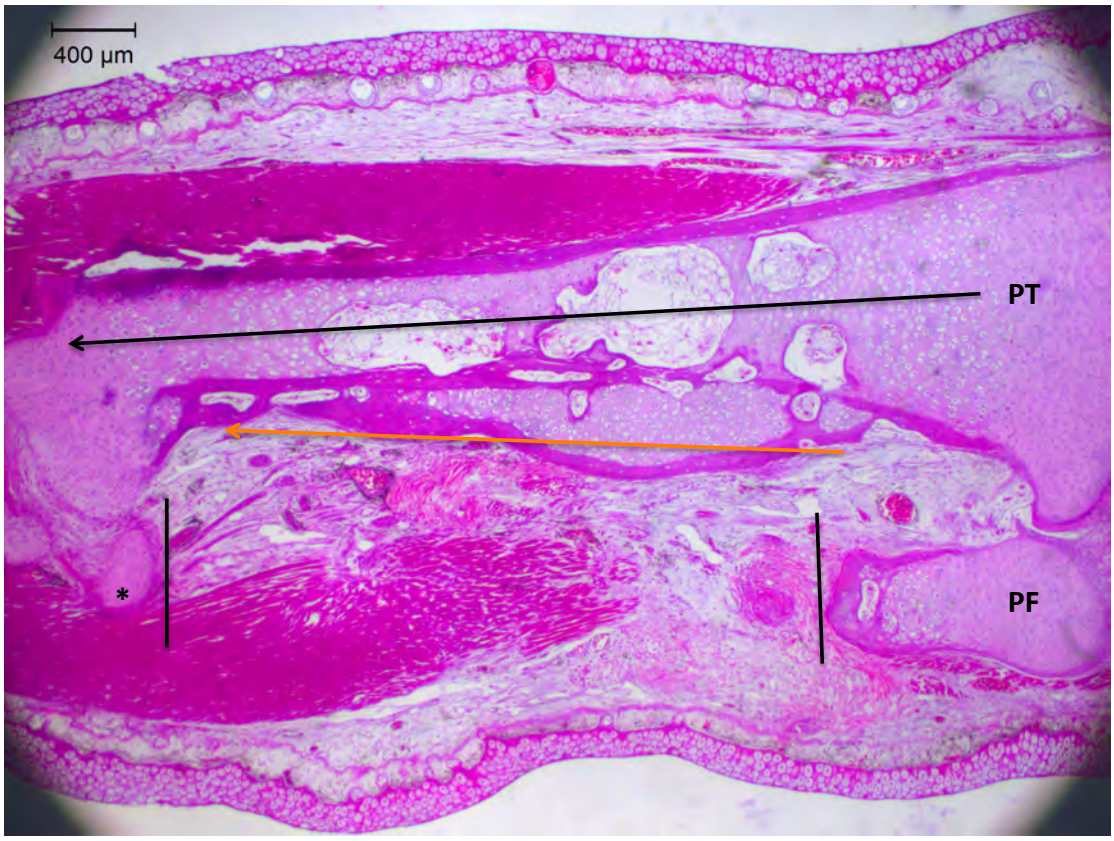
Tibia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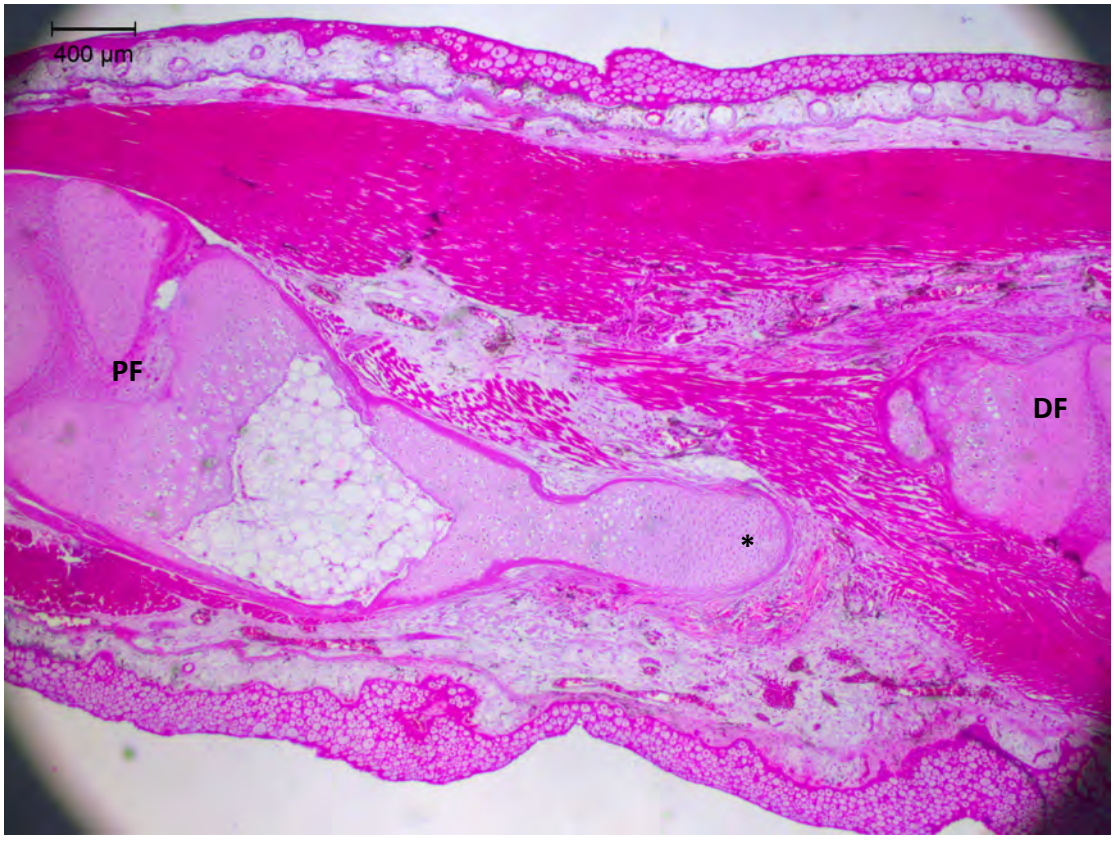
PT
PF
*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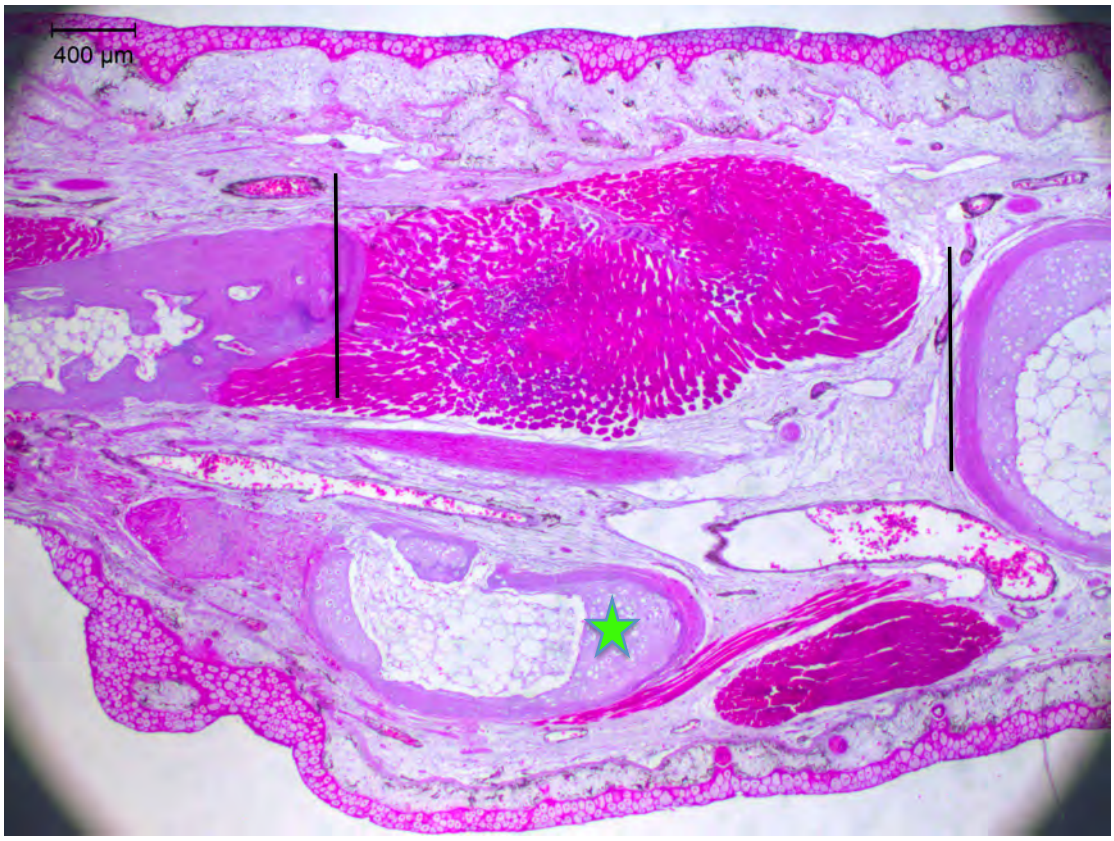
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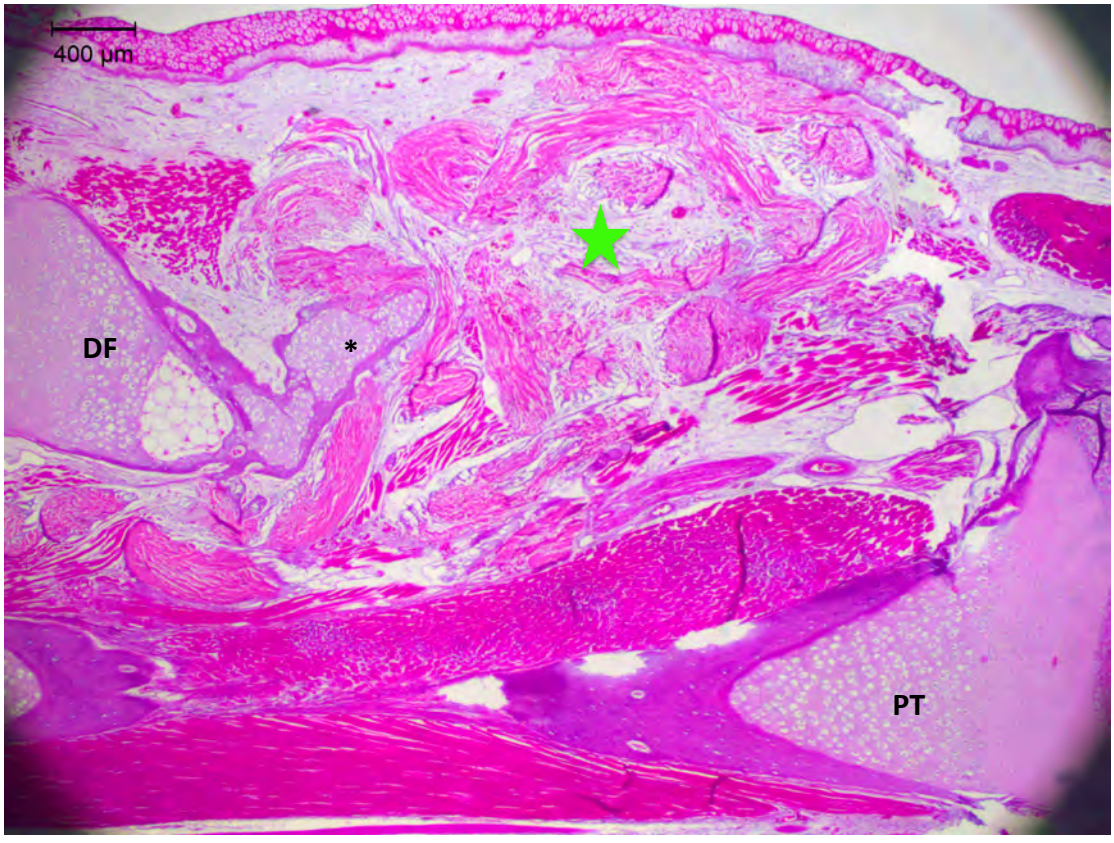
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